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Integration of various stacking processes in carrier ampholyte-based capillary electrophoresis

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Abstract

Field-enhanced sample stacking, field-enhanced sample injection as well as electrokinetic supercharging have been successfully integrated in carrier ampholyte-based capillary electrophoresis. Through the analysis of different test sample mixtures, it has been shown that the carrier ampholyte-based background electrolytes, in spite of their very low conductivity, allow efficient online preconcentration of analytes by field-amplified techniques. Sensitivity enhancement factors of the same magnitude as those usually encountered with classical conductive background electrolytes have been obtained in such carrier ampholyte-based buffers. Depending on the online preconcentration method that has been integrated, sensitivity enhancement factors between 50 and several thousands have been reached.

Keywords: Carrier ampholytes; CABCE; FESS; FESI; EKS

1. Introduction

The use of isoelectric background electrolytes (BGEs) for capillary zone electrophoresis (CZE) has been introduced by Hjerten et al. in 1995 [1]. The use of such BGE in CZE allows the application of high electric field strength without inducing any significant Joule heating in the system because of their low conductivity.

So far, different kinds of compounds have been used as low conductivity buffers in CZE. Through different studies, amino acid-based buffers have been proven to be a valuable alternative to classical BGEs. As an example, we can mention that the tryptic peptide map of bovine β-casein was performed in less than 10 min in an aspartic acid-based buffer while 80 min were necessary in an 80 mM classical phosphate buffer [2]. Cysteic acid [3], histidine or the His-Gly dipeptide [4] and iminodiacetic acid [5–7] have also been used as low conductivity buffers in CZE.

The main drawback of amino acids is that only few of them exhibit a sufficient buffering capacity to be used as BGE in CZE [8]. In this context, we have been interested in the development of isoelectric buffers composed of narrow pH cut of carrier ampholytes (CAs). CAs are usually used as a very heterogeneous mixture in isoelectric focusing (IEF). In such a mixture, covering, for example a pH 3–10 range, a very high number of compounds can be found [9–11]. To obtain solutions containing only CAs with close isoelectric points (p*I*), we fractionated a wide pH range mixture of CAs by preparative IEF in a granulated gel [12]. With this fractionation technique, between 25 and 30 narrow pH cut fractions of CAs have been obtained. As the CAs are the sole buffering species in each narrow pH cut, the fraction pH should be rather close to the p*I* of the contained CAs. As a consequence, each fraction should present a low conductivity value. This has been confirmed by the physicochemical characterization of the 25 narrow pH cuts obtained

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from a wide pH range mixture of home made CAs [12]. In addition to a low conductivity, it has been shown that most of the obtained CA fractions (20 over 25) were presenting a suitable buffering capacity to be used as BGE in capillary electrophoresis (CE). Then, it has been proven that proteins as well as peptides can be rapidly and efficiently separated by carrier ampholyte-based capillary electrophoresis (CABCE) [13,14]. The coupling between CABCE and electrospray ionization mass spectrometry (ESI-MS) has also been implemented in the context of protein tryptic digest analysis [13]. In order to further broaden the application range of the CA-based BGEs, we have evaluated in the present work the integration of online preconcentration methods in CABCE for the analysis of low abundant analytes.

In CE, depending on the used BGE, different online preconcentration methods can be chosen. The most commonly used are the field-enhanced sample stacking (FESS) (with or without water removal), field-enhanced sample injection (FESI), transient isotachophoresis (tITP), dynamic pH junction and sweeping. The first three quoted are based on electromigration phenomena while the latter one allows preconcentration by non-covalent interactions. In dynamic pH junction, pH differences along the separation path are used to enhance the sensitivity. These methods have been well described in recent review articles [15–18]. When used individually, sensitivity enhancement factors (SEFs) ranging from few tens to few thousands can be achieved. However, some of the described methodologies may be used in combination to reach higher SEFs. For example, FESI has been successfully combined with sweeping a few years ago [19]. In that case, extremely high SEFs (between 550,000 and 900,000) have been obtained for 1-naphthylamine and laudanosine, respectively. In other reports, FESI has been coupled to tITP for the online preconcentration of several kinds of samples; small cations, sodium dodecyl sulfate protein complexes or DNA fragments [20–22]. This method has been named by the authors electrokinetic supercharging (EKS).

Here, FESS, FESI and EKS have been considered as the low conductivity of the CA-based electrolyte might represent *a priori* a severe drawback to field-enhanced methods. All these methods are based on the same preconcentration principle but differ by the way in which the sample is introduced in the capillary.

While a hydrodynamic injection is used in FESS, an electrokinetic injection is performed in FESI. If the electrophoretic mobility of the analyte is high enough, it generally allows the injection of more analyte molecules in the capillary. Thus, a higher sensitivity is usually obtained with FESI [23,24]. Also, as the sample plug length inside the separation capillary is shorter after FESI than after FESS, the available length for CZE separation is longer. The separation resolution is consequently favored with FESI. For the same reason, if an electroosmotic flow (EOF) exists in the capillary, problems linked to the co-existence of two different EOFs are reduced if FESI is integrated [25].

Concerning the mechanism of FESS and FESI, analyte preconcentration occurs at the boundary between the sample and the BGE zones where a sharp change in analyte velocity exists. It is created because the conductivity difference between the two zones induces a difference in the local electric field strengths. The process can be well understood if we consider two of the basic laws that regulate the electrophoretic processes.

Besides electroneutrality and mass balance which have to be respected, the modified Ohm's law and the Kohlrausch regulating function (KRF) rule the electromigration phenomena [15]. The modified Ohm's law governs the local electric fields in the different sections of the separation capillary. Indeed, for the current density to remain constant over the whole capillary, the electric fields are adjusted according to the specific conductivity of each zone. The modified Ohm's law is expressed by the following equation:

$$j = \sigma E \tag{1}$$

where j ($\mathrm{A\,m^{-2}}$) represents the current density, σ ($\Omega^{-1}\,\mathrm{m^{-1}}$) the specific zone conductivity and E ($\mathrm{V\,m^{-1}}$) the electric field strength.

As the modified Ohm's law regulates in CZE the local electric fields in the different zones of the capillary, it determines if a boundary is stabilized or not. Indeed, if an ionic species, during electrophoresis, leaves by diffusion its zone in the direction of electromigration and reaches another zone of higher conductivity. Following the modified Ohm's law, it will be submitted to a lower electric field strength and will consequently be caught up by its own zone, resulting in a stabilized boundary. On the contrary, if it reaches a zone with a lower conductivity, it will be accelerated because of an increase in electric field strength and will not be caught up by its zone. This is the case of a non-stabilized and diffuse boundary. The concept of diffuse or stabilized boundary is of first importance when dealing with stacking mechanisms. For example, if the sample matrix presents a lower conductivity than the BGE, the analyte velocity will be higher in the sample zone than in the BGE zone and the stacking process will be initiated at the considered boundary.

Still, this is only one part of the process because the KRF also has to be considered. As can be seen from Eq. (2), its numerical value is set by the contents of the separation capillary before the application of an electric field and remains constant during the whole electrophoretic process. The KRF thus prescribes that the ionic compounds (i) of the sample will, during their migration, adapt their concentration (c_i) according to their mobility (u_i) to the KRF ω constant value of the BGE

$$\omega = \sum_{i}^{n} \frac{c_\mathrm{i}}{|u_\mathrm{i}|} \tag{2}$$

By analogy, when entering into the initial sample zone, the BGE ions will adapt their concentration following their electrophoretic mobilities to the KRF value of the sample.

Taking into account the fundamentals of electrophoresis, it appears that FESS or FESI are favored by the use of buffers presenting both a high conductivity and a high loading capacity. However, in this paper, we demonstrate that preconcentration techniques based on field amplification can be efficiently integrated in CA-based BGEs in spite of their low conductivity.

Table 1
pH and conductivity of the CA-based BGEs

CA-based BGE no.	pH	Conductivity (S m^{-1})
6	3.5	0.024
10	4.5	0.018
22	8	0.013

2. Materials and methods

2.1. Chemicals

Buffer and sample solutions were prepared with water purified using a Milli-Q system from Millipore (Bedford, MA, USA). All solutions were passed through 0.45 μm filters (Nacalai Tesque, Kyoto, Japan) prior to use. Peptide standards, cytochrome *c*, lithium chloride, sodium 2-naphthoate, sodium salicylate and sodium 2-naphtalenesulfonate were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Each chemical has first been dissolved in water.

2.2. CA-based buffer preparation

A wide pH range mixture (pH range 4–9) of Servalyts from Coger (Paris, France), has been fractionated into 28 fractions according to Busnel et al. [12]. The CA fraction stock solutions, eluted with water from the Sephadex beads were collected after the fractionation by preparative IEF and simply diluted in distilled water before their use as BGE in CE. Considering the different dilutions performed, each narrow pH cut of CAs has been used in CE at approximately 1% (w/v) of ampholytes. Three CA-based BGEs have been considered in this work, their pH and conductivity are summarized in Table 1.

2.3. Tryptic digestion

Horse heart cytochrome *c* (2 mg mL^{-1}) was dissolved in a 75-mM ammonium hydrogencarbonate buffer (pH 7.7) and heated for 5 min. Then, trypsin was added to the protein solution (enzyme-to-protein ratio 1:100, w/w) and the digestion was carried out overnight at 37 °C.

2.4. Instrumentation

The CABCE experiments were carried out with an HP^{3D}CE apparatus (Agilent, Waldbronn, Germany) equipped with a diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. Data were handled by HP Chemstation software. Fused silica capillaries from Polymicro were provided by Photon Lines (Paris, France) and coated with hydroxypropylcellulose (HPC) in the laboratory following the procedure described earlier by Shen and Smith [26]. The capillaries were of 50 μm I.D. and 375 μm O.D. and of various lengths as indicated in the captions to the figures. Between different separations in the same BGE, a buffer rinse was only performed. Water and the new buffer were successively flushed into the capillary when different buffers have been used in the same capillary. Hydrodynamic injection or electrokinetic injection as stated in the corresponding figure captions has been used for sample injection. SEFs were calculated according to the following equation:

$$\mathrm{SEF} = \frac{h' f}{h} \tag{3}$$

where h' is the peak height of the preconcentrated analyte, h the peak height of the non-preconcentrated analyte while detected after a conventional injection and f the dilution factor. For all experiments, the dilution medium has been water.

3. Results and discussion

In this study, we have assessed the performances of CA-based buffers for the online preconcentration of analytes. For this evaluation, we considered three sample mixtures. The first one is a mixture of 2-naphthalenesulfonate, salicylate and 2-naphthoate. The second one is a mixture of three peptides (bradykinin, angiotensin I and angiotensin II) and the last one is the tryptic digest of horse heart cytochrome *c*. While the organic compounds have been analyzed at pH 8 under their anionic form, the peptides have been separated at acidic pH under their cationic form. We chose to use HPC-coated capillaries in order to suppress the EOF. This permitted to focus only on the properties of the CA-based BGEs for online preconcentration methods. Indeed, if an EOF is present in the system, when implementing FESS, the heterogeneity existing between the EOF values in the sample and in the BGE zone induces extra peak broadening [25].

3.1. FESS in CA-based BGEs

First, the simplest online preconcentration method has been considered. This method is simply integrated by hydrodynamically injecting a large sample volume as compared to a classical injection of few nanoliters. As described in Section 1, the preconcentration mechanism is based on a sudden decrease of the analyte velocity at the boundary between the initial sample zone and the BGE zone. This stacking is due to the conductivity difference between the two zones considered and also to the existing difference between their KRF values. In fact, even if it has not yet been properly demonstrated, we can figure out that the decrease in velocity allows the stacking phenomenon to occur and that the KRF heterogeneity set the maximum sensitivity enhancement that can be reached. In our case, the difference between the conductivity of the samples and BGE is about 10. This conductivity ratio has been estimated by current measurements in CE (data not shown). Thus, the conductivity difference between the sample and the BGE is rather low and the question raised was to know if this would prevent the stacking capabilities or not. First, the small organic compound mixture has been considered. Fig. 1 shows the electropherogram A obtained when a highly concentrated sample is analyzed by CABCE under conventional (no stacking) conditions and the one that can be acquired when a 100 times diluted sample is analyzed under optimized FESS conditions (trace B). While SEF around 90 can be calculated for each of the analyzed compound, we can see that the integration of FESS does not induce a dramatic decrease of resolution.

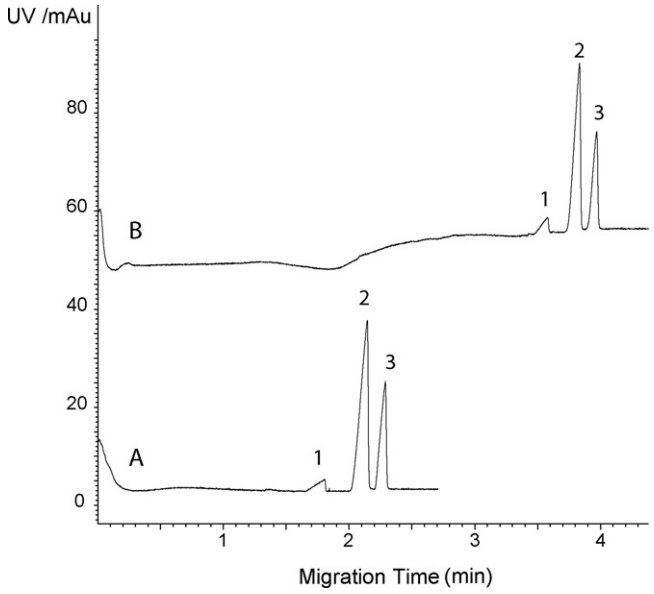

Fig. 1. CABCE separation of salicylate, 2-NPS and 2-naphthoate at pH 8 (narrow pH cut No. 22). HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D., voltage: −30 kV, current: −3 μA, temperature: 25 °C, UV absorbance at 227 nm. Peak order 1–3: salicylate, 2-NPS, 2-naphthoate. (A) 30 mbar 2 s (3 nL). Sample: [salicylate] = [2-NPS] = 0.67 mM, [2-naphthoate] = 0.34 mM. (B) 30 mbar 120 s (168 nL). Sample: [salicylate] = [2-NPS] = 6.7 μM, [2-naphthoate] = 3.4 μM.

Indeed, Fig. 2 reports the evolution of the resolution between salicylate and 2-NPS and between 2-NPS and 2-naphthoate as a function of the sample injection time. If we compare the resolution observed when the non-diluted sample (horizontal lines) is injected in the capillary by a few nanoliter injection (3 nL) to that observed when a large volume of the 100 times diluted sample

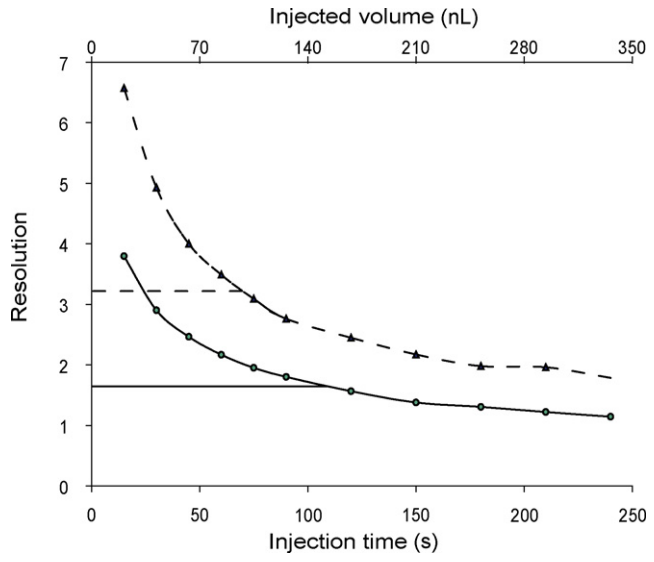

Fig. 2. Resolution as a function of the injection time in FESS: organic compound mixture. Dashed line: resolution between salicylate and 2-NPS; continuous line: resolution between 2-NPS and 2-naphthoate. HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D. pH 8 (narrow pH cut No. 22), voltage: −30 kV, current: −3 μA, temperature: 25 °C, UV absorbance at 227 nm. Hydrodynamic injection. Sample: [salicylate] = [2-NPS] = 6.7 μM, [2-naphthoate] = 3.4 μM.

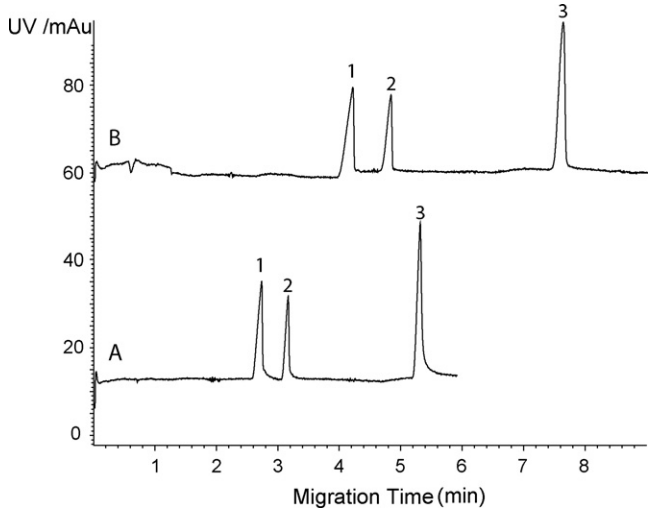

Fig. 3. CABCE separation of bradykinin, angiotensin I and angiotensin II at pH 4.5 (narrow pH cut No. 10). HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D., voltage: 30 kV, current: 2.1 μA, temperature: 25 °C, UV absorbance at 222 nm. Peak order 1–3: bradykinin, angiotensin I, angiotensin II. (A) 30 mbar 2 s (3 nL). Sample: [bradykinin] = [angiotensin I] = [angiotensin II] = 0.5 g L^{-1}. (B) 30 mbar 60 s (84 nL). Sample: [bradykinin] = [angiotensin I] = [angiotensin II] = 0.01 g L^{-1}.

is injected in the capillary. We can see that an 84-nL injection does not deteriorate the resolution of the separation. Also, if we consider the maximum volume that can be injected while maintaining baseline resolution (resolution > 1.6), it appears that as much as 168 nL (case of Fig. 1) can be injected in the 687 nL (total volume) capillary.

After assessing FESS for small organic compounds, we used a more acidic CA-based BGE presenting a pH of 4.5 for the analysis of the standard peptide mixture. We can observe in Fig. 3 the electropherograms corresponding to the analysis of the non-diluted peptide sample under conventional conditions (A) and to the 50 times diluted sample when FESS is integrated (B). SEF between 38 and 46 are achieved depending on the peptides; the fastest peptide being the most preconcentrated one. Also, as previously studied for the small organic compound mixture, the evolution of the resolution between the peptides as a function of the injection time is reported in Fig. 4. As shown in Fig. 3, we can perform an injection representing a volume of 84 nL, without deteriorating the resolution between the analyzed peptides.

In both cases, small organic compound or peptide analysis, the maximum available electric field strength (857 V/cm) has been applied. Because of the low conductivity of the CA-based BGEs, the induced current (in absolute value) ranged from 2.1 to 3 μA. Thus, in addition to the absence of Joule heating, FESS can be used efficiently in CABCE to increase moderately the sensitivity without decreasing the resolution of the analysis. What is most important here is that the reported SEFs are of the same magnitude as those usually reached by FESS in more conductive classical BGEs. For each injection time value, three measurements have been made. For the small organic compound mixture, migration times showed a relative standard deviation (RSD) below 1.5% while peak heights showed RSD below 4%. Regarding the analysis of the peptides, migration times showed

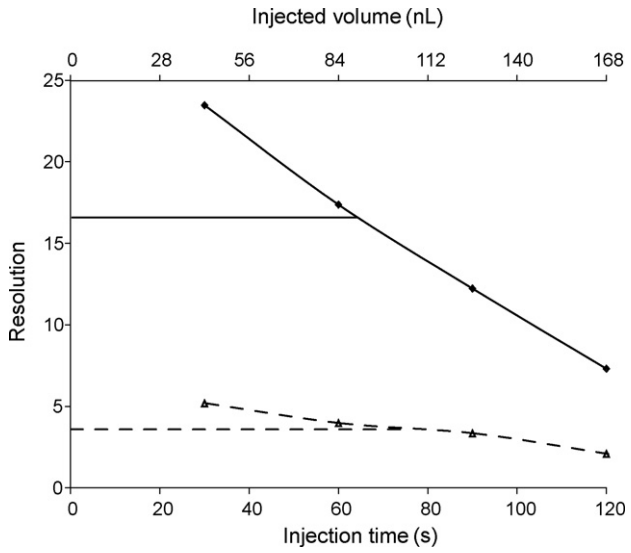

Fig. 4. Resolution as a function of the injection time in FESS: peptide mixture. Dashed line: resolution between bradykinin and angiotensin I; continuous line: resolution between angiotensin I and angiotensin II. HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D. pH 4.5 (narrow pH cut No. 10), voltage: 30 kV, current: 2.1 μA, temperature: 25 °C, UV absorbance at 222 nm. Hydrodynamic injection. Sample: [bradykinin] = [angiotensin I] = [angiotensin II] = 0.01 g L^{-1}.

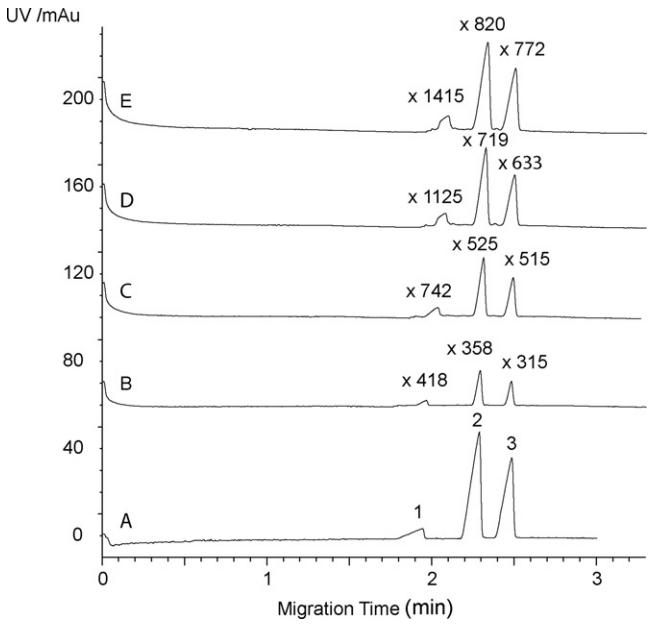

Fig. 5. FESI preconcentration of salicylate, 2-NPS and 2-naphthoate at pH 8 (narrow pH cut No. 22). HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D., voltage: −30 kV, current: −3 μA, temperature: 25 °C, UV absorbance at 227 nm. Peak order 1–3: salicylate, 2-NPS, 2-naphthoate. (A) Injection: 30 mbar 2 s (3 nL). Sample: [salicylate] = [2-NPS] = 0.67 mM, [2-naphthoate] = 0.34 mM. (B) Injection: −4 kV 30 s. One thousand times diluted sample: [salicylate] = [2-NPS] = 0.67 μM, [2-naphthoate] = 0.34 μM. (C) Injection: −4 kV 60 s; sample as in B. (D) Injection: −4 kV 90 s; sample as in B. (E) Injection: −4 kV 120 s; sample as in B.

a RSD below 4.5% and peak heights below 7.5%. Thus, the repeatability appears to be lower for the peptides than for the small organic compounds. Given that the samples were simply diluted in water, it could be explained by the higher dependence on pH of the peptide electrophoretic mobilities.

3.2. FESI in CA-based BGEs

FESS is the simplest method to use to enhance the sensitivity of a CZE analysis, but the SEF that can be reached is limited and can hardly be superior to 100. To achieve a higher preconcentration, FESI can be integrated. FESI simply consists in injecting the sample by a large electrokinetic injection in an appropriate BGE, providing SEF values of several hundred or even more than one thousand in some cases. It is one of the most effective preconcentration methods usable in CE. As previously explained, the mechanism allowing the stacking of the analytes in FESI is the same as in FESS. However, as compared to the hydrodynamic injection used in FESS, a higher amount of molecules can be introduced in the capillary with large electrokinetic injections. It has to be stated that this is true provided that the considered analyte presents a sufficiently high electrophoretic mobility.

The same sample test mixtures and CA-based BGEs, as previously used, have been used to assess the FESI performances. In Fig. 5, the small organic compound mixture is considered. Trace A reports the results obtained when the non-diluted sample is analyzed without any preconcentration step. The other experiments have been carried out with the 1000 times diluted sample under zone sharpening conditions. Above each peak, the calculated SEFs (see Section 2 for definition) are indicated. When the performed electrokinetic injection is larger than −4 kV 60 s, the shape of the salicylate peak, which is no longer triangular, clearly suggests an overloading. Nevertheless, a SEF above 1000 can be reached. Unlike the salicylate peak, the peaks corresponding to the two other analytes remain triangular whatever the injection conditions. For these analytes, the SEFs that can be reached are less important than the one obtained for salicylate. This can be explained by the lower electrophoretic mobilities of 2-NPS and 2-naphthoate.

Then, FESI was also applied to the peptide test mixture analysis. The obtained electropherograms are shown in Fig. 6. It first appears that the use of FESI in CABCE brings to the fore the presence of impurities in the peptide sample. But fortunately, it does not hinder the detection of the peptides that are still well separated. As for the small organic compound mixture, the fastest analyte is the one which can be the most concentrated. Indeed, in the best case, bradykinin presents a SEF value close to 600 while angiotensin I and II are presenting SEFs of 449 and 329, respectively. Also, as compared to the small organic compound mixture, the preconcentration appears to be less effective for the peptides. This can be explained by their lower mobilities. Nevertheless, having demonstrated that fast anionic analytes can be efficiently preconcentrated by FESI in CABCE, it is shown in Fig. 6 that a similar approach could be applied to cationic analytes of moderate electrophoretic mobilities. To summarize, these analyses demonstrate that in spite of the low conductivity of the CA-based BGE, FESI, as FESS can be efficiently integrated in CABCE to strongly increase the sensitivity of the analysis. Also, it is demonstrated that several CA-based BGEs presenting different pH are suitable for the inte-

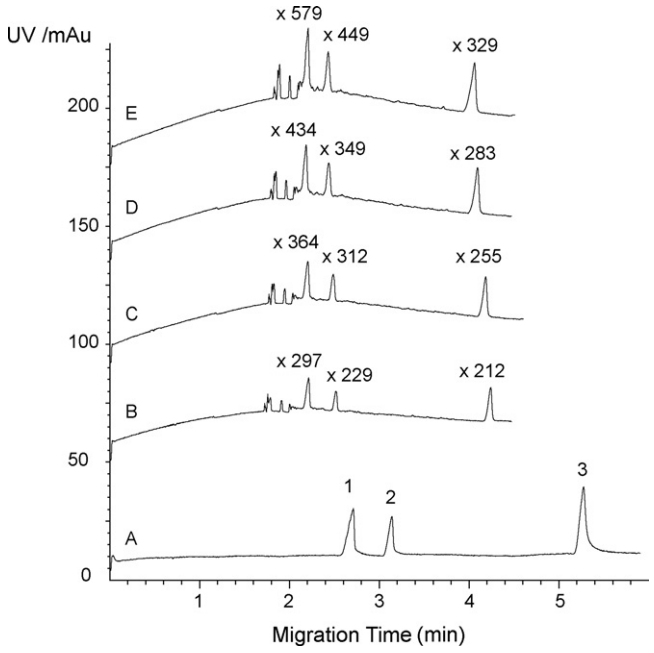

Fig. 6. FESI preconcentration of bradykinin, angiotensin I and angiotensin II at pH 4.5 (narrow pH cut No. 10). HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D., voltage: 30 kV, current: 2.1 μA, temperature: 25 °C, UV absorbance at 222 nm. Peak order 1–3: bradykinin, angiotensin I, angiotensin II. (A) Injection: 30 mbar 2 s (3 nL). Sample: [bradykinin] = [angiotensin I] = [angiotensin II] = 0.5 g L^{-1}. (B) Injection: 18 kV 90 s. Sample: [bradykinin] = [angiotensin I] = [angiotensin II] = 1 mg L^{-1}. (C) Injection: 22 kV 90 s; sample as in B. (D) Injection: 26 kV 90 s; sample as in B. (E) Injection: 30 kV 90 s; sample as in B.

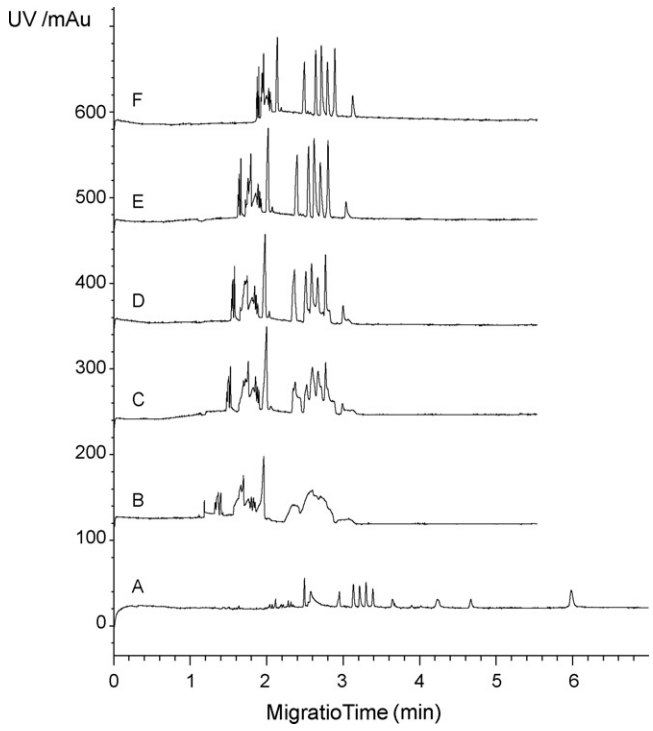

Fig. 7. Integration of EKS in CABCE. HPC-coated capillary, total/effective length 35/26.5 cm × 50 μm I.D., narrow pH cut No. 6 (pH 3.5), voltage: 30 kV, current: 4 μA, temperature 25 °C, UV absorbance at 222 nm. Sample: tryptic digest of cytochrome *c* at 25 μM (A) or 250 nM (B–E). Sample injection: 30 mbar 2 s (A) or 20 kV 120 s (B–F). LE injection: 30 mbar 2 s. (A) No LE plug injection; (B) no LE plug injection; (C) LE: 50 mM LiCl; (D) LE: 100 mM LiCl; (E) LE: 150 mM LiCl; (E) LE: 200 mM LiCl.

gration of FESS and FESI, and that anions, as cations, can be efficiently preconcentrated. This versatility may be very useful when both separation and sample preconcentration have to be simultaneously optimized.

If we consider the low conductivity of the CA-based BGEs, the results of this study may appear surprising. However, as we have seen in Section 1, not only the conductivity of a BGE for stacking is important but also the loading capacity. In this context, we have shown in a previous study that the loading capacity of the CA-based BGEs is rather high in comparison to one of the classical BGEs, either isoelectric or not [27]. However more studies need to be conducted, the high value of this parameter for narrow pH cuts of CAs may explain their suitability for stacking integration.

3.3. EKS in CA-based BGEs

After assessing the capabilities of CA-based BGEs to integrate FESS or FESI on sample test mixtures, we wanted to know if these methodologies could be used for the analysis of a more complex sample. To this end, the tryptic digest of horse heart cytochrome *c* has been considered. The results obtained are reported in Fig. 7. Electropherogram A corresponds to the case of a sample concentrated enough to be analyzed without the integration of any preconcentration step. The other electropherograms have been obtained with a sample 100 times diluted in water. This corresponds approximately to a concentration of 500 nM. In a first try, we have tried to integrate FESI (trace B, Fig. 7). As can be seen, it allows the detection of the peptides however the obtained resolution is very low. This is particularly true for the peptides presenting a low mobility.

In a previous study, we have shown that tITP can be easily integrated in CABCE to improve the sensitivity of protein analysis [28]. Thus, CA-based BGEs should also be buffers that are compatible with the integration of EKS, a methodology using both, electrokinetic injection and t-ITP. After assessing the possibility to integrate EKS using sodium as the leading ion, we chose to work with lithium because it presents an electrophoretic mobility that is more compatible with one of the analyzed peptides. Indeed, by using lithium as the leading ion, the tITP step is of benefit to most of the peptides. As can be seen in Fig. 7, only the three less mobile peptides are not affected by the used preconcentration methodology and are consequently not detected. If we consider the electropherograms B–F, the pattern evolution as a function of the lithium chloride concentration is rather characteristic of the presence of a tITP step. Indeed, we can first observe that the migration time is linearly increasing with the leading ion concentration. Then, it appears that the closer the electrophoretic mobility of the peptide from the lithium electrophoretic mobility, the more efficient the tITP step is. Indeed, the fast peptides are preconcentrated even at low leading ion concentration and the SEFs that can be reached for those are much higher than those obtained in the case of slow peptides. Depending on the

considered peptide, SEF values between several hundreds and several thousands can be reached by the integration of EKS in CA-based BGEs. Also, as it has been said before, it appears in this preliminary study that the very slow peptides, presenting an electrophoretic mobility below $15\,cm^2\,V^{-1}\,s^{-1}$, cannot be efficiently preconcentrated under the chosen conditions.

4. Conclusion

The present study demonstrates that in spite of their low conductivity, sample stacking (FESS and FESI) can be used in CABCE in order to enhance the sensitivity of the analysis. Both anions and cations can be preconcentrated in such buffers and several narrow pH cuts of CA can be used. When considering the separation optimization of a given mixture, this versatility may be really helpful to develop a suitable and sensitive method. It has also been shown that EKS has to be positively considered when rather complex mixtures have to be analyzed in CABCE. Indeed, it allows both a strong sensitivity enhancement and the maintainability of high resolution.

From a theoretical point of view, the capabilities of CA-based BGEs for FESS and FESI integration are rather surprising given the low conductivity of these solutions. However, even if this should be confirmed by other studies, it could certainly be explained by the value of their loading capacity that has previously been found to be considerably high. Future studies on the importance of the conductivity and/or the KRF of a BGE for the integration of stacking processes have to be carried out. While this kind of study would bring toward a better understanding of these complex mechanisms, it would also allow the development of more efficient preconcentration methodologies.

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